

# **Effects of vitamin D<sub>3</sub> and calcium supplementation on serum levels of tocopherols, retinol, and specific vitamin D metabolites**

Weiwen Chai,<sup>1</sup> Roberd M. Bostick,<sup>2,3</sup> Thomas U. Ahearn,<sup>4</sup> Adrian A. Franke,<sup>1</sup>  
Laurie J. Custer,<sup>1</sup> Robert V. Cooney<sup>5</sup>

<sup>1</sup> Epidemiology Program, Cancer Research Center of Hawaii, University of Hawaii, 1236  
Lauhala Street, Honolulu, HI 96813.

<sup>2</sup> Department of Epidemiology, Rollins School of Public Health, Emory University, 1518 Clifton  
Road Northeast, Atlanta, GA 30322.

<sup>3</sup> Winship Cancer Institute, Emory University, Atlanta, GA 30322

<sup>4</sup> Nutrition and Health Sciences Program, Graduate Division of Biological and Biomedical  
Sciences, Emory University, 1462 Clifton Road, Suite 314  
Atlanta, Georgia 30322.

<sup>5</sup> Office of Public Health Sciences, John A. Burns School of Medicine, University of Hawaii at  
Manoa, 1960 East-West Road, Honolulu, HI 96822

**ABSTRACT**

$\gamma$ -Tocopherol ( $\gamma$ T) protects against DNA-damaging effects of nitrogen oxides, yet its physiologic regulation *in vivo* is unknown. Observational studies indicate inverse associations of 25[OH]-vitamin D with  $\gamma$ T and leptin. To determine whether vitamin D<sub>3</sub> supplementation alters levels of lipid-soluble micronutrients, serum samples (N=85 subjects) from a randomized, double-blind, placebo-controlled clinical trial of vitamin D<sub>3</sub> (800 IU) and calcium (2 g) alone and in combination were analyzed for lipid micronutrients and specific vitamin D metabolites at baseline and after 6-months of supplementation. Serum 25[OH]-vitaminD<sub>3</sub> levels increased 55% ( $P < 0.0001$ ) and 48% ( $P = 0.0005$ ), whereas 25[OH]-vitaminD<sub>2</sub> levels decreased by 48% ( $P = 0.26$ ) and 21% ( $P = 0.36$ ) in the vitamin D<sub>3</sub> and vitamin D<sub>3</sub> plus calcium groups, respectively. At baseline,  $\gamma$ T levels were inversely associated with 25[OH]D ( $r = -0.31$ ,  $P = 0.004$ ). With vitamin D<sub>3</sub> plus calcium treatment, serum  $\alpha$ -tocopherol decreased 14% ( $P = 0.04$ ), while similar changes in  $\gamma$ T (19% decrease,  $P = 0.14$ ) were observed. No significant effects were observed for D<sub>3</sub> supplementation on leptin or retinol levels. These results are consistent with the hypothesis that vitamin D<sub>3</sub> +/- calcium affects serum tocopherol and 25[OH]D<sub>2</sub> levels, however, studies utilizing larger populations are warranted.

## INTRODUCTION

The tocopherols are important agents in preventing cellular oxidative damage, and are postulated to protect against the development of cancer and other aging-related diseases (1, 2). Although  $\alpha$ -tocopherol ( $\alpha$ T) has the highest level of vitamin E bioactivity amongst the tocopherols (3) and, consequently, has been the target of most animal and epidemiologic research over the years, more recent findings have pointed to the other tocopherols, particularly  $\gamma$ -tocopherol ( $\gamma$ T), as playing significant roles in inhibiting the development of cancer in animal models (2). Epidemiological evidence, while limited, also suggests an inverse association of  $\gamma$ T with certain cancers (4, 5) and heart disease (6).  $\gamma$ T has been identified as a key cellular antioxidant that specifically blocks damage to cells resulting from the enzymatic production of nitric oxide (NO), a mediator of inflammation through its oxidation products (7, 8). Conversely,  $\alpha$ T appears to be a primary reactant against cellular oxygen-based oxidants, but lacks anti-inflammatory activity (9). Together the two forms of tocopherol protect cells from the major forms of endogenous oxidative assault. The assumption that serum tocopherol levels reflect dietary intake is valid only in cases of severe deficiency or heavy supplement use (10); hence, the physiologic determinants of serum levels of the tocopherols may be key to elucidating their potential functions for either reducing/promoting disease or serving as markers of risk. In particular, serum levels of  $\gamma$ T appear to be unrelated to dietary intake and may reflect to a large extent the level of chronic inflammation as suggested by its positive associations with circulating C-reactive protein and urinary isoprostane (11), thereby complicating the epidemiologic interpretation of its associations with diseases.

Vitamin D is associated with lower risk for cancer, primarily colorectal (12, 13), cardiovascular disease (14), and all-cause mortality (15). Vitamin D deficiency may result in chronic inflammation and decreased immune function (16). It was observed previously that serum 25(OH)-vitamin D (25[OH]D, as the sum of 25[OH]D<sub>2</sub> + 25[OH]D<sub>3</sub>), and  $\gamma$ T were significantly inversely associated with one another and in turn associated with all-cause mortality (11). In that same study, a positive association of circulating  $\alpha$ T with 25[OH]D was observed. It was hypothesized that deficiency of vitamin D may lead to a state of chronic inflammation resulting in elevated  $\gamma$ T levels which protect against NO-mediated damage, thereby compensating against some of the deleterious consequences of vitamin D deficiency. However, in previous studies (17, 18), it was also observed that both body mass index (BMI) and leptin (an adipocytokine produced predominantly by white adipose tissue) were inversely associated with serum 25[OH]D and positively associated with  $\gamma$ T levels. This suggests the possibility that the inverse association between  $\gamma$ T and 25[OH]D may be incidental to obesity and not directly related to vitamin D status. Also, there is *in vitro* evidence supporting regulation of leptin by vitamin D (19) and regulation of vitamin D by leptin (20), but definitive *in vivo* evidence is lacking.

Circulating retinol levels are reported to rise in winter and decline in summer, inverse to changes predicted for vitamin D (7), suggesting a possible interaction and/or compensation between vitamins D and A. Research evidence in animals (21) and humans (22) suggests that high doses of vitamin A may antagonize vitamin D actions. Support for an interaction between vitamins A and D is also provided in a recent nested case-control study of colorectal cancer by Jenab, et al. (23) where the retinol-colon

cancer association was inverse among individuals with low vitamin D but direct among individuals with higher vitamin D levels. To our knowledge, there are no published data on the influence of vitamin D supplementation on circulating vitamin A and tocopherol levels. The primary objective of the current study was, therefore, to examine the effects of vitamin D<sub>3</sub> and/or calcium supplementation on changes of serum levels of  $\alpha$ T,  $\gamma$ T, retinol (vitamin A), and leptin levels. In addition, we sought to determine whether supplemental D<sub>3</sub> and calcium, alone or in combination, affect serum levels of specific vitamin D metabolites such as 25[OH]D<sub>2</sub>, 25[OH]D<sub>3</sub>, and vitamins D<sub>2</sub> and D<sub>3</sub>.

## METHODS

*Study population and protocol:* This study utilized stored serum samples from a previous pilot, randomized, double-blind, placebo-controlled, 6-month clinical trial that used a 2 x 2 factorial design to test the effects of calcium and/or vitamin D on biomarkers of risk for colon cancer (24). In brief, 92 adults aged 30 to 75 years, in general health, capable of informed consent, with a history of at least one pathology-confirmed adenomatous colorectal polyp within the past 36 months, no contraindications to calcium or vitamin D supplementation or rectal biopsy procedures and no medical conditions, habits, or medication usage that would otherwise interfere with the study, and taking  $\geq$  80% of their study pills during 1-month placebo run-in period, were recruited from the patient population attending the Digestive Diseases Clinic at the Emory Clinic, Emory University. The Institutional Review Boards of Emory University and the University of Hawaii approved the current study protocol.

At baseline, eligible participants were randomly assigned to the following four treatment groups: a placebo control group (placebo), a 2.0 g elemental calcium (as calcium carbonate in equal doses twice daily) supplementation group (calcium), an 800 IU [as 400 IU cholecalciferol (D<sub>3</sub>) twice daily] vitamin D<sub>3</sub> supplementation group (vitamin D<sub>3</sub>), and a calcium plus vitamin D<sub>3</sub> supplementation group (calcium plus vitamin D<sub>3</sub>) taking 2.0 g elemental calcium plus 800 IU vitamin D<sub>3</sub> daily. The corresponding supplement and placebo pills were identical in size, appearance, and taste. The placebo was free of calcium, magnesium, vitamin D, and chelating agents. Additional details on the rationale for the doses and forms of calcium and vitamin D<sub>3</sub> supplements were described previously (24).

The treatment period was 6 months, and participants attended follow-up visits at 2 and 6 months after randomization and were contacted by telephone between the second and final follow-up visits. Pill-taking adherence was assessed by questionnaire, interview, and pill count. Participants were instructed to remain on their usual diet and not take any nutritional supplements not in use on entry into the study. Blood was collected at baseline (randomization) and at study completion (6-month follow-up), and diet was assessed with a semi-quantitative food frequency questionnaire (25). A total of 85 subjects (21-22 per group) completing the study with blood samples at baseline and at six months were available for analysis and utilized in the current study.

*Sample analyses:* Serum levels of vitamins D<sub>2</sub> and D<sub>3</sub>, 25[OH]D<sub>2</sub>, and 25[OH]D<sub>3</sub> were analyzed using liquid chromatography orbitrap mass spectrometry. An aliquot of 0.3 mL serum was mixed with 0.3 mL methanol, 2 mL hexane, and 15 µL of a methanolic

solution containing D<sub>3</sub>-*d*<sub>6</sub> and 25[OH]D<sub>3</sub>-*d*<sub>6</sub> (Medical Isotopes, Pelham, NH). After centrifugation, the hexane layer was removed and subsequently evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 0.15 mL methanol and 20 µL extract was injected into HPLC (model Accela, ThermoFisher, San Jose, CA). Separation was conducted on an Ascentis Express C18 column (150 x 3.0 mm i.d., 2.7 µm; Supelco, Park Bellefonte, PA) and a pre-filter cartridge (2.1 mm i.d., 0.2 µm; ThermoFisher, San Jose, CA) with a mobile phase gradient consisting of isopropanol/water/methanol/acetonitrile (0/12/ 69.6/18.4 [vol/vol/vol] for 3.5 min, 0/0/60/40 for 4.5 min, 55/0/10/35 for 6 min, and 0/12/69.6/18.4 for 5.1 min, successively). The flow rate was kept at 0.7 mL/min. After electrospray ionization, the orbitrap mass spectrometer (model Exactive, ThermoFisher, San Jose, CA), with exact mass determination to five decimal places, was operated at the following masses for quantitating the respective vitamin D metabolites: m/z 379.336/ 380.339/ 397.346/ 398.349 for D<sub>2</sub>, m/z 367.336/ 385.346/ 386.349 for D<sub>3</sub>, m/z 377.320/ 378.324/ 395.330/ 396.335/ 413.341/ 414.341 for 25[OH]D<sub>2</sub>, m/z 365.320/ 366.323/ 383.330/ 384.333/ 401.341/ 402.341 for 25[OH]D<sub>3</sub>, m/z 391.383 for D<sub>3</sub>-*d*<sub>6</sub>, and m/z 389.368 for 25[OH]D<sub>3</sub>-*d*<sub>6</sub>.

Serum concentrations of total 25[OH]D were also measured according to the manufacturer's directions using an immunoassay kit (Immunodiagnostic Systems, Ltd. Fountain Hills, AZ). Leptin immunoassay kits purchased from R&D System (Minneapolis, MN) were used to analyze serum leptin levels according to the manufacturer's instructions. Serum retinol and tocopherol levels were analyzed by HPLC

with pre-column electrochemical oxidation and post-column UV detection, as previously described in detail (11, 26).

*Statistical Analysis:* Treatment groups (placebo, calcium, vitamin D<sub>3</sub>, calcium plus vitamin D<sub>3</sub>) were assessed for comparability of characteristics at baseline and at final follow-up by Fisher's exact test for categorical variables and ANOVA for continuous variables.

Primary analyses were based on assigned treatment at the time of randomization regardless of adherence status (intent-to-treat analysis). Means of outcome variables were calculated for each treatment group for the baseline and 6-month follow-up visits. Treatment effects were evaluated by assessing the differences in the outcome variables from baseline to the 6-month follow-up visit between participants in each active treatment group and the placebo group by a repeated-measures linear MIXED effects model. The model included the intercept, follow-up visit effects (baseline and follow-up), and interactions between treatment groups and the follow-up visit effect (the absolute treatment effect). To provide perspective on the magnitude of the treatment effects, we also calculated proportional treatment effects, defined as (absolute treatment effect / treatment group baseline) x 100% (e.g., a proportional effect of 56% would mean a 56% increase in the active treatment group relative to the placebo group). Because the treatment groups were balanced on potentially influential factors (participant characteristics and dietary intakes of certain nutrients) at baseline, no adjustment was made for other covariates in the primary intent-to-treat analyses. Spearman correlation was used to assess the associations between analyte levels. SAS software (SAS Institute,



Cary, North Carolina) was used for analyses. All tests were two sided, and  $P < 0.05$  was considered statistically significant.

## RESULTS

### *Characteristics of study participants*

The four treatment groups did not differ significantly with respect to participant characteristics measured at baseline (Table 1, based on a total of 92 originally randomized subjects). The mean age of participants was  $60.5 \pm 7.9$  (SD) years, 64% were men, 71% were white, 88% were current non-smokers, and 82% were overweight or obese. The mean values of BMI were  $30.1 \pm 6.1$  kg/m<sup>2</sup> at baseline and  $30.2 \pm 6.2$  kg/m<sup>2</sup> at 6-month follow-up ( $p = 0.38$ ). Baseline values of  $\gamma$ T for 30.6 % of the 85 subjects who completed the trial were considered hyper-gammatocopherolemic (11) with levels  $> 2.5$   $\mu$ g/ml. This was comparable to what was observed previously for male control subjects in another study (11), where 28.9% were found to have elevated  $\gamma$ T. After the six-month intervention 22.4% of the 85 subjects in all four treatment groups were observed to be hyper-gammatocopherolemic. Adherence to visit attendance averaged 92% and did not differ significantly among the four treatment groups. On average, at least 80% of pills were taken by 93% of participants at the first follow-up visit and 84% at the final follow up visit. There were no treatment complications.

*Effects of supplemental calcium and/or vitamin D<sub>3</sub> on specific serum vitamin D metabolites.*

Serum specific vitamin D measures are shown in Table 2. At baseline, there were no significant differences between the four treatment groups in 25[OH]D<sub>3</sub>, 25[OH]D<sub>2</sub>, and vitamins D<sub>3</sub> and D<sub>2</sub> levels. Serum levels of 25[OH]D<sub>3</sub> significantly increased by approximately 50% at the end of 6-month's follow-up in the treatment groups that received supplemental D<sub>3</sub> alone ( $P < 0.0001$ ) or combined with calcium ( $P = 0.0005$ ) relative to placebo. There were no statistically significant treatment effects on serum levels of 25[OH]D<sub>2</sub>, vitamin D<sub>2</sub>, or vitamin D<sub>3</sub>. However, relative to placebo, 25[OH]D<sub>2</sub> levels decreased by 48% in the supplemental vitamin D<sub>3</sub> group ( $P = 0.26$ ) and 21% in the vitamin D<sub>3</sub> plus calcium group ( $P = 0.36$ ), and 16% higher ( $P = 0.75$ ) in the calcium group after six months. Serum vitamin D<sub>3</sub> levels were not significantly changed relative to placebo after supplementation, but were observed to be 34% higher ( $P = 0.08$ ) among individuals supplemented with D<sub>3</sub> alone and 17% higher ( $P = 0.36$ ) in those who received D<sub>3</sub> plus calcium, relative to baseline.

*Effects of supplemental calcium and/or vitamin D<sub>3</sub> on serum tocopherols, retinol, and leptin.*

Baseline serum levels of 25[OH]D were significantly inversely correlated with  $\gamma$ T ( $r = -0.31$ ,  $P = 0.004$ ) but were not significantly correlated with  $\alpha$ T ( $r = 0.12$ ,  $P = 0.29$ ). Serum  $\alpha$ T and  $\gamma$ T levels were not significantly correlated with each other at baseline ( $r = -0.10$ ,  $P = 0.38$ ).

Mean serum levels of  $\alpha$ T,  $\gamma$ T, retinol, and leptin for each group at baseline and after six months supplementation are shown in Table 3. While both vitamin D<sub>3</sub> intervention groups had lower  $\gamma$ T levels after six months supplementation with vitamin D<sub>3</sub> relative to baseline, the absolute treatment effect, which also factors in changes in the placebo group, were not statistically significant. Serum  $\alpha$ T and  $\gamma$ T levels decreased by 14% ( $P = 0.04$ ) and 19% ( $P = 0.14$ ), respectively, in the calcium plus vitamin D<sub>3</sub> group relative to placebo when analyzed by absolute treatment effect. There also appeared to be a trend for decreasing serum  $\gamma$ T levels as indicated by the relative treatment effects (calcium group, -3%,  $P = 0.85$ ; vitamin D<sub>3</sub> group, -8%,  $P = 0.57$ ; calcium plus vitamin D<sub>3</sub> group, -19%,  $P = 0.14$ ). As shown in Figure 1, when all vitamin D<sub>3</sub>-supplemented subjects were combined and stratified by baseline 25[OH]D level, there was a clear trend toward both increased 25[OH]D and lower serum  $\gamma$ T levels after six months of vitamin D<sub>3</sub> treatment across all quartiles, consistent with the inverse association between serum  $\gamma$ T and 25[OH]D observed both at baseline and after treatment.

Although lower retinol levels were observed in supplemental D<sub>3</sub> +/- calcium-treated subjects (Table 3), slightly higher retinol levels were observed in subjects treated with vitamin D<sub>3</sub> alone, indicating no consistent effect of vitamin D<sub>3</sub> supplementation on retinol. In the current study 82% of study participants were overweight or obese and their weight remained constant over the 6-month experimental period (mean BMI: baseline,  $30.1 \pm 6.1 \text{ kg/m}^2$ ; 6 months,  $30.2 \pm 6.2 \text{ kg/m}^2$ ). Baseline leptin values were highly correlated with BMI ( $r = 0.67$ ,  $P < 0.0001$ ) and significantly correlated with  $\gamma$ T ( $r = 0.24$ ,  $P = 0.03$ ). No significant changes in leptin levels (Table 3) were observed for any treatment group, consistent with the stable BMI levels observed. The calcium + vitamin

D<sub>3</sub> group, relative to the other treatment groups, did have borderline elevated leptin levels at baseline, which remained high at the six-month follow-up.

## DISCUSSION

Several studies (27-29) reported that supplemental D<sub>2</sub> or D<sub>3</sub> increases circulating levels of 25[OH]D, an indicator of vitamin D nutritional status in humans, and vitamin D<sub>3</sub> is considered to be the more biologically potent form (30, 31). As expected, this randomized clinical trial of daily supplementation with 800 IU vitamin D<sub>3</sub> demonstrated that increased serum 25[OH]D<sub>3</sub> was responsible for the increase in total 25[OH]D observed, but vitamin D<sub>3</sub> supplementation did not increase 25[OH]D<sub>2</sub> levels. Indeed, lower 25[OH]D<sub>2</sub> levels were observed suggesting that a possible competing effect on 25[OH]D<sub>2</sub> should be considered in larger studies of D<sub>3</sub> supplementation. In contrast, Holick et al. (27) reported that a 1,000 IU dose of supplemental D<sub>2</sub> daily for 11 weeks did not negatively affect serum 25[OH]D<sub>3</sub> levels. In the present study, using a modest dose of D<sub>3</sub> (800 IU per day) in the upper range of current official recommendations (32), serum levels of 25[OH]D<sub>3</sub> increased by approximately 8 ng/ml in individuals who received D<sub>3</sub> +/- calcium, going from an average of approximately 19 ng/ml (inadequate) to approximately 27 ng/ml (near sufficient), whereas mean 25(OH) D<sub>2</sub> levels were lower after supplementation by approximately one ng/ml in those supplemented with D<sub>3</sub> +/- calcium. Vitamin D<sub>3</sub>, synthesized in the skin under the influence of solar ultraviolet light, is the primary source of vitamin D (other than dietary supplements) for most people (33). Thus, the clinical significance of reduced circulating 25[OH]D<sub>2</sub> levels due to vitamin D<sub>3</sub> supplementation may not be relevant since 25[OH]D<sub>3</sub> (derived from D<sub>3</sub>) is

considered the predominant form in the circulation; nevertheless, the physiological significance of 25[OH]D<sub>2</sub> independent of 25[OH]D<sub>3</sub> remains unclear at present.

Results from the present pilot study support, but do not prove, the hypothesis that vitamin D<sub>3</sub> supplementation, alone or in combination with calcium, alters serum  $\alpha$ T and  $\gamma$ T levels. At baseline, serum 25[OH]D and  $\gamma$ T were significantly inversely correlated, whereas  $\alpha$ T was positively, but not statistically significantly, associated with 25[OH]D, consistent with previously published findings (11). Lower  $\alpha$ T and a trend towards lower  $\gamma$ T were observed after D<sub>3</sub> supplementation (Table 3 and Figure 1); however, the sample size was too small to detect the estimated absolute treatment effect on  $\gamma$ T. The demonstration of a possible causal relationship between sub-optimal 25(OH) D<sub>3</sub> levels and circulating  $\gamma$ T is particularly important, as the means by which  $\gamma$ T levels are regulated physiologically are not known. Given the observed associations between 25(OH) and  $\gamma$ T in this and a previous study (11), vitamin D would be predicted to account for approximately 5-20% of the variability in circulating  $\gamma$ T if a causal relationship existed. The observed borderline significant changes in response to D<sub>3</sub> supplementation (about 12% overall for the combined groups receiving D<sub>3</sub>) are consistent with such a postulated effect, where supplementation with D<sub>3</sub> changed vitamin D status from deficiency to near adequate levels. As in a previous study (11), we also observed a significant inverse association between 25[OH]D and  $\gamma$ T at baseline and after supplementation (Figure 3). The previous observation that this inverse correlation was strongest in African Americans and Latinos (11) takes on greater significance in light of the current study and suggests that future efforts might be targeted towards a specifically vitamin D-deficient population to fully establish whether there is a causal relationship.

The previously reported positive correlation of serum  $\alpha$ T and 25[OH]D levels (11) was also observed in the baseline levels of the participants in the present study (although it was not statistically significant); however, vitamin D<sub>3</sub> supplementation in the current study clearly did not increase circulating  $\alpha$ T, as  $\alpha$ T levels were significantly lower after supplementation in the calcium plus vitamin D<sub>3</sub> group. The positive association for  $\alpha$ T in the previous study may have been due to the multi-ethnic population studied (11) and/or the confounding effects of  $\alpha$ T supplementation in that population. This effect was not tested in the current study, as there was no significant correlation between  $\alpha$ T and  $\gamma$ T levels at baseline and no  $\alpha$ T was provided as a supplement. Another explanation may be that vitamin D deficiency does indeed cause both tocopherols to rise but  $\alpha$ T is depleted to a greater extent because of the increased oxidation associated with an inflammatory state resulting from vitamin D deficiency. Evidence for such a mechanism is provided in cell culture studies where it was observed that inducing oxidative stress causes  $\gamma$ T to rise in relation to  $\alpha$ T; however, if NO synthesis and a corresponding oxidative assault are inhibited, then levels of both tocopherols would rise in response to the inflammatory trigger (34). In a separate analysis of the subjects used in the current study (35), biomarkers of inflammation were observed to be lower in vitamin D<sub>3</sub>-treated subjects, consistent with the hypothesis that tocopherols were lower as a result of decreased inflammation. The potentially interactive effects of calcium with vitamin D<sub>3</sub> seen for tocopherols in the present study are intriguing and warrant further investigation to determine the mechanism of action.

Previous reports indicated that serum 25[OH]D levels were inversely associated with BMI (17, 18), suggesting a possible influence of obesity and its associated effects on

leptin on metabolic pathways of vitamin D and /or its precursors. In the current study the majority (82%) of study participants were overweight or obese and their weight remained constant over the 6-month experimental period (mean BMI: baseline,  $30.1 \pm 6.1 \text{ kg/m}^2$ ; 6 months,  $30.2 \pm 6.2 \text{ kg/m}^2$ ), as did serum leptin levels (Table 3). Therefore, our results suggest that: 1) supplementing 800 IU of vitamin D<sub>3</sub> daily for 6 months effectively raised vitamin D status via increased circulating 25[OH]D<sub>3</sub> levels in this middle aged/elderly, overweight study population; 2) supplemental D<sub>3</sub> may depress 25[OH]D<sub>2</sub> levels (46% in the vitamin D<sub>3</sub> group and 21% in the calcium plus vitamin D<sub>3</sub> group); and 3) supplemental 25[OH]D<sub>3</sub> had no significant impact on serum leptin levels. In addition, we found no evidence for increased levels of circulating vitamin D<sub>3</sub> in overweight individuals, indicating that weight and/or leptin did not significantly affect the conversion of circulating vitamin D<sub>3</sub> to 25[OH]D<sub>3</sub>.

The strengths of the present study included the randomized, double-blind, placebo-controlled trial design; high protocol adherence by study participants; and that it is the first study to examine both the independent and combined effects of calcium and vitamin D<sub>3</sub> on serum antioxidant micronutrients and specific vitamin D metabolites. Limitations of the current study included the relatively small sample size in each treatment group and the high BMI levels of most subjects. However, the findings from this pilot study are nevertheless novel, consistent with previous observational and cell culture data that point to a possible causal relationship between vitamin D deficiency and circulating tocopherol levels, and provide important data for the future designs of studies to assess the effects of vitamin D<sub>3</sub> supplementation on biochemical and nutritional parameters.

In summary, in addition to the statistically significant increase in serum 25[OH]D<sub>3</sub> and αT levels and a significant baseline association between 25[OH]D and γT, the current results suggest that a modest dose of 800 IU of supplemental vitamin D<sub>3</sub>, alone or in combination with 2 g of elemental calcium daily for 6 months, may affect circulating 25[OH]D<sub>2</sub> and γT levels as well, but appears to have no appreciable effects on retinol or leptin levels. The current study cannot rule out the possibility that significantly higher levels of 25[OH]D might impact retinol levels since vitamin D levels were generally low in the study population and the intervention raised circulating levels only moderately. Recommendations for optimal vitamin D levels in nutrition remain controversial and many authorities recommend substantially higher levels clinically for disease prevention (36, 37) which may have additional impacts on lipid and inflammatory biomarkers. No evidence was found for leptin blocking the conversion of D<sub>3</sub> to 25[OH]D<sub>3</sub> *in vivo* or for reductions in leptin levels by vitamin D<sub>3</sub> supplementation. Future larger clinical trials are needed to confirm these observations and to explore the effects of higher doses of vitamin D +/- calcium on physiologically relevant antioxidants and vitamins.

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**Corresponding author and requests for reprints:**

Robert V. Cooney, Office of Public Health Sciences, John A. Burns School of Medicine,  
University of Hawaii-Manoa, 1960 East-West Road, Honolulu, HI 96822; tel: 808-956-  
5775; fax: 808-956-5818; rvcooney@hawaii.edu

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Table 1. Selected baseline<sup>a</sup> characteristics and dietary intakes of the study participants (n = 92).

	Treatment Group				P <sup>b</sup>
	Placebo (n = 23)	Calcium (n = 23)	Vitamin D <sub>3</sub> (n = 23)	Calcium +Vitamin D <sub>3</sub> (n = 23)	
<b><u>Characteristics</u></b>					
Age, y	58.5 (8.2)	61.9 (8.2)	60.2 (8.1)	62.1 (7.5)	0.39
Men (%)	70	70	70	70	1.00
White (%)	74	83	65	61	0.39
College graduate (%)	65	61	57	44	0.53
Current smoke (%)	13	9	13	13	0.96
Take multivitamin (%)	30	30	26	39	0.86
Body mass index, kg/m <sup>2</sup>	30.6 (7.2)	29.4 (5.5)	28.9 (5.6)	31.6 (6.0)	0.44
Waist to hip ratio	0.93 (0.08)	0.92 (0.09)	0.92 (0.10)	0.98 (0.11)	0.17
<b><u>Mean dietary intakes</u></b>					
Energy, kcal/d	1,596 (528)	1,788 (691)	1,848 (821)	1,845 (752)	0.59
Calcium, mg/d <sup>c</sup>	618 (308)	746 (335)	843 (526)	824 (714)	0.41
Vitamin D, IU/d <sup>c</sup>	277 (230)	336 (202)	360 (317)	415 (316)	0.40
Fat, g/d	67 (32)	72 (35)	70 (32)	74 (28)	0.59
Alcohol, g/d	9 (14)	11 (15)	14 (18)	10 (20)	0.84

<sup>a</sup> Data are given as mean (SD) unless otherwise specified.

<sup>b</sup> By Fisher's exact  $\chi^2$  test for categorical variables or ANOVA for continues variables.

<sup>c</sup> Diet plus supplements.

Table 2. Serum levels of specific vitamin D measurements at baseline and after 6 months of treatment.

Analyte Treatment Group	Baseline			6-month Follow-up			Absolute Rx Effect <sup>b</sup>		Proportional Rx Effect <sup>c</sup> (%)
	N	Mean (SE) <sup>a</sup>	P <sup>d</sup>	N	Mean (SE) <sup>a</sup>	P <sup>d</sup>	Mean (SE) <sup>a</sup>	P <sup>d</sup>	
<u>25(OH)D<sub>3</sub></u> (ng/mL)									
Placebo	20	18.8 (1.9)		21	17.2 (1.9)		0		-
Calcium	21	23.4 (1.9)	0.10	21	22.5 (2.0)	0.06	0.8 (2.5)	0.74	3.4
Vitamin D <sub>3</sub>	21	19.4 (1.9)	0.84	21	28.3 (2.0)	0.0001	10.7 (2.4)	<0.0001	55.2
Calcium + Vitamin D <sub>3</sub>	21	18.8 (1.9)	0.98	18	26.0 (2.0)	0.002	9.0 (2.5)	0.0005	47.9
<u>25(OH)D<sub>2</sub></u> (ng/mL)									
Placebo	20	2.5 (1.3)		21	2.4 (1.3)		0		-
Calcium	21	1.9 (1.3)	0.76	21	2.1 (1.4)	0.89	0.3 (1.0)	0.75	15.8
Vitamin D <sub>3</sub>	21	2.3 (1.3)	0.93	21	1.2 (1.3)	0.52	-1.1 (1.0)	0.26	-47.8
Calcium + Vitamin D <sub>3</sub>	21	4.3 (1.3)	0.35	18	3.3 (1.4)	0.64	-0.9 (1.0)	0.36	-20.9
<u>Vitamin D<sub>3</sub></u> (ng/mL)									
Placebo	20	13.2 (1.5)		21	12.3 (1.6)		0		-
Calcium	21	12.7 (1.5)	0.78	21	12.7 (1.7)	0.87	0.9 (2.4)	0.70	7.1
Vitamin D <sub>3</sub>	21	12.5 (1.5)	0.81	21	15.8 (1.6)	0.12	4.2 (2.3)	0.08	33.6
Calcium + Vitamin D <sub>3</sub>	21	12.6 (1.5)	0.76	18	13.8 (1.6)	0.50	2.1 (2.3)	0.36	16.7
<u>Vitamin D<sub>2</sub></u> (ng/mL)									
Placebo	20	0.44 (0.16)		21	0.42 (0.16)		0		-
Calcium	21	0.31 (0.16)	0.56	21	0.34 (0.17)	0.74	0.06 (0.19)	0.77	19.4
Vitamin D <sub>3</sub>	21	0.64 (0.16)	0.39	21	0.54 (0.17)	0.60	-0.08 (0.19)	0.68	-12.5
Calcium + Vitamin D <sub>3</sub>	21	0.49 (0.16)	0.84	18	0.45 (0.17)	0.91	-0.02 (0.19)	0.91	-4.1

<sup>a</sup>Least-squares means are used.

<sup>b</sup>Absolute treatment (Rx) effect = [(treatment group follow-up) – (treatment group baseline)] – [(placebo group follow-up) – (placebo group baseline)].

<sup>c</sup>Proportional treatment (Rx) effect = (absolute treatment effect / treatment group baseline) x 100% (e.g., a proportional effect of 55% would mean that a 55% increase in the active treatment group relative to the placebo group).

<sup>d</sup>P value for difference between each active treatment group and the placebo group from repeated measures MIXED model.



Table 3. Serum levels of  $\alpha$ -tocopherol,  $\gamma$ -tocopherol, retinol, and leptin at baseline and after 6 months of treatment.

Analyte Treatment Group	Baseline			6-month Follow-up			Absolute Rx Effect <sup>b</sup>		Proportional Rx Effect <sup>c</sup> (%)
	N	Mean (SE) <sup>a</sup>	P <sup>d</sup>	N	Mean (SE) <sup>a</sup>	P <sup>d</sup>	Mean (SE) <sup>a</sup>	P <sup>d</sup>	
<u><math>\alpha</math>-Tocopherol</u> (ng/mL)									
Placebo	21	13.4 (1.0)		21	14.3 (1.0)		0		-
Calcium	21	14.3 (1.0)	0.57	21	14.2 (1.1)	0.93	-1.0 (1.1)	0.36	-7.0
Vitamin D <sub>3</sub>	22	13.7 (1.0)	0.87	22	13.5 (1.0)	0.59	-1.0 (1.0)	0.32	-7.3
Calcium + Vitamin D <sub>3</sub>	21	15.5 (1.0)	0.15	21	14.3 (1.0)	0.98	-2.2 (1.0)	0.04	-14.2
<u><math>\gamma</math>-Tocopherol</u> (ng/mL)									
Placebo	21	2.38 (0.25)		21	2.39 (0.25)		0		-
Calcium	21	1.97 (0.26)	0.24	21	1.93 (0.26)	0.20	-0.05 (0.28)	0.85	-2.9
Vitamin D <sub>3</sub>	22	2.01 (0.25)	0.28	22	1.87 (0.26)	0.14	-0.15 (0.27)	0.57	-7.5
Calcium + Vitamin D <sub>3</sub>	21	2.13 (0.26)	0.47	21	1.74 (0.26)	0.07	-0.40 (0.26)	0.14	-18.8
<u>Trans Retinol</u> (ng/mL)									
Placebo	21	548.4 (33.9)		21	513.5 (34.4)		0		-
Calcium	21	597.4 (33.9)	0.31	21	583.1 (36.1)	0.17	20.5 (41.3)	0.62	3.4
Vitamin D <sub>3</sub>	22	573.3 (33.1)	0.60	22	549.7 (34.0)	0.46	11.3 (39.8)	0.78	2.0
Calcium + Vitamin D <sub>3</sub>	21	567.4 (33.9)	0.69	21	500.0 (33.9)	0.78	-32.4 (39.4)	0.41	-5.7
<u>Leptin</u> (ng/mL)									
Placebo	21	17.2 (4.5)		21	17.6 (4.5)		0		-
Calcium	21	18.1 (4.5)	0.89	21	17.3 (4.5)	0.97	-1.1 (2.7)	0.68	-6.0
Vitamin D <sub>3</sub>	22	14.9 (4.4)	0.72	22	16.8 (4.4)	0.91	1.5 (2.5)	0.54	10.1
Calcium + Vitamin D <sub>3</sub>	21	26.3 (4.5)	0.15	21	24.1 (4.5)	0.30	-2.5 (2.5)	0.33	-9.5

<sup>a</sup> Least-squares means are used.

<sup>b</sup> Absolute treatment (Rx) effect = [(treatment group follow-up) – (treatment group baseline)] – [(placebo group follow-up) – (placebo group baseline)].

<sup>c</sup> Proportional treatment (Rx) effect = (absolute treatment effect / treatment group baseline) x 100% (e.g., a proportional effect of 55% would mean that a 55% increase in the active treatment group relative to the placebo group).

<sup>d</sup> P value for difference between each active treatment group and the placebo group from repeated measures MIXED model.

